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[71] 申请人 王思勉
地址 130012 吉林省长春市长春高新技术产业开发区天河街 72 号
[72] 发明人 王思勉

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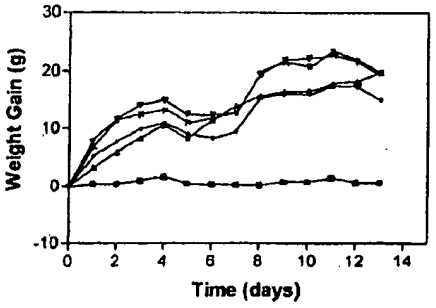
[54] 发明名称 聚乙二醇人生长激素缀合物及其制备方法及其药物用途

[57] 摘要

本发明涉及一种聚乙二醇人生长激素缀合物及其制备方法及药物用途，本发明为每个 hGH 分子与单一 PEG 分子偶联的缀合物，防止 hGH 在体内的迅速降解，形成的聚合物比 hGH 具有更好的稳定性，及明显延长体内的半衰期。



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1、一种聚乙二醇人生长激素缀合物，其特征是一种被连接到一种聚乙二醇(PEG)上的人生长激素(hGH)，该PEG的分子量为15kDa到50kDa，其中每个hGH分子与单一PEG分子偶联。

2、根据权利要求1所述的聚乙二醇人生长激素缀合物，其特征在于：聚乙二醇（PEG）有一个单一的具反应活性的醛基。

3、根据权利要求1所述的聚乙二醇人生长激素缀合物，其特征在于：人生长激素（hGH）通过N端α氨基被连接到PEG上。

4、根据权利要求1中所述的聚乙二醇人生长激素缀合物，其特征在于： PEG的分子量为20kDa到40kDa。

5、根据权利要求1中所述的聚乙二醇人生长激素缀合物，其特征在于：人生长激素指基因工程表达的人生长激素分子。

6、一种制备聚乙二醇人生长激素缀合物的方法，其特征在于：

(a)烷基化反应：向pH为3-9的4℃溶液中加入hGH和已活化的PEG醛， hGH与PEG醛摩尔比为1：0.1到1：1，PEG醛的平均分子量为15 kDa 到50kDa；

(b)反应产物的分离和纯化：在该反应过程中，蛋白质改性的程度用分子筛凝胶色谱进行监测，用含有0.1M氯化钠、pH为6.5的50mM磷酸钠在0.4毫升/分钟的流速下进行洗脱；5小时后，经分子筛凝胶色谱法的分析表明所有的hGH已经基本上转化成为N一末端聚乙二醇化的衍生物的形式；

随后用无菌水将该反应混合物总共稀释五倍，并且将其加到一个离子交换柱上，再用 pH 为 7.0 的 50mM 磷酸钠缓冲溶液饱和该柱；在 1 毫升/分钟的流速下向该柱中装填反应混合物，并且用三倍柱体积的同样的缓冲溶液洗脱未反应的 PEG 的醛；采用线性梯度洗脱 N 一末端聚乙二醇化的 hGH，该线性梯度采用从 0%至 100%的 50mM 磷酸钠，其 pH 为 7.5、含 0.2M 氯化钠，进行 100 分钟，含有该 hGH 缀合物的部分被合并、浓缩并且无菌过滤。

7、根据权利要求 6 中所述的制备聚乙二醇人生长激素缀合物的方法，其特征在于：在烷基化反应时，hGH：PEG 范围：摩尔比为 1：0.15 到 1：0.6，pH 范围 为 4.5 到 7。

8、一种聚乙二醇人生长激素缀合物在制备治疗儿童或成人生长激素缺乏症的药物中的应用。

聚乙二醇人生长激素缀合物及其制备方法及其药物用途

技术领域

本发明涉及蛋白质与聚乙二醇 PEG 缀合物，以及其制备方法和药物用途。

背景技术

Ross Clark 等于 1996 年，The Journal of Biological Chemistry 271: 21969-21977, 1996，首次公开了用 PEG 偶联人生长激素的方法和药理活性的研究结果，他们应用的 PEG 的分子量为 5kDa， PEG-GH 缀合物的半衰期明显延长，可以每 5 天注射一次，使患者使用更方便。但此缀合产物的每个 GH 分子上偶联了 1-7 个 PEG 分子，缀合物产物是一种混合物，偶联位点不确定，每种成分无法定量，也无法完全分离，使得批与批之间缀合物的产品成分差异性较大，无法应用于临床。

各种天然的和重组的蛋白质都具有医药用途。一旦将它们纯化、分离并配成药品，可以将它们经非肠道途径进行给药，而用于不同的医疗适应症，然而，非肠道途径给予的蛋白质可以是免疫原性的、可以是相对不溶于水的且可以具有较短的药理半衰期。因此，难以使所述的蛋白质在患者体内达到有治疗作用的血药浓度。

通过将所述的蛋白质与聚合物如 PEG 偶联后可以克服这些难

题。Davis 等在美国专利 4179337 中公开了将 PEG 与蛋白质如酶和胰岛素偶联以获得具有低于原始蛋白质的免疫原性作用并仍可保持其基本比例的药理活性的缀合物的技术方案。Katre 等在美国专利 4766106 和 4917888 中还公开了通过聚合物偶联使蛋白质增溶的技术方案。同样，可以将 PEG 和其它聚合物与蛋白质偶联以便降低免疫原性并延长半衰期。

随着生物医学及基因工程技术的发展和深入，大量的药用 PEG 化蛋白质被制备、生产并应用于临床。现在已应用于临床的药用 PEG 化蛋白质有 PEG-干扰素、PEG-粒细胞集落刺激因子等。

人生长激素是人脑垂体分泌的一种蛋白类激素，能促进人体充分利用各方面的能量和原料合成蛋白质，促进营养中的钙沉积在骨骼，增加身体细胞的大小和数量，使骨骼和内脏器官成比例增长。1958 年国外科学家就从人脑垂体中提取出生长激素用以治疗侏儒症，人类垂体重约 0.5g，含 GH 量极微，每个垂体仅含 GH 5-10mg。由于垂体来源的限制，临床应用和研究进展极其缓慢。1979 年，DNA 生物技术使得人类 GH 的 DNA 顺序密码在大肠杆菌中的表达成为可能，从而生产了用 DNA 重组技术人工合成的重组人生长激素 rhGH。随着生产技术的不断提高，rhGH 产品的质量及产量均有很大提高。1985 年，用基因工程技术人工合成的重组人生长激素开始应用于临床，给患者带来福音。由于 rhGH 在体内的半衰期短，患者必须每天注射一次，使用非常不方便。

发明内容

根据hGH 的N端暴露的特点，选择性的将PEG分子偶联到hGH 的 N 端，而不与 hGH 蛋白上的其它自由氨基反应，此方法生产的 PEG-hGH 为每个 hGH 分子与单一 PEG 分子偶联，最终产物成分单一，易于分离纯化，收率高。PEG-hGH 的半衰期可达 5-7 天，减少用药频率，每 5-7 天注射一次，方便患者使用。

本发明是一种被连接到一种聚乙二醇上的人生长激素，该 PEG 的分子量为 15kDa 到 50kDa，其中每个 hGH 分子与单一 PEG 分子偶联：

本发明不限于特定分子量的 PEG 分子，但它们都具有一个单一的具有反应活性的醛基。在一个实施方案中，所述的聚乙二醇是 PEG_{40000}。在一个优选的实施方案中，用尿烷键使所述的 hGH 与所述的 PEG_{40000} 分子连接。

本发明制备聚乙二醇人生长激素缀合物的方法：

(a)烷基化反应：向 pH 为 3-9 的 4℃溶液中加入 hGH 和已活化的 PEG 醛， hGH 与 PEG 醛摩尔比为 1：0.1 到 1：1，PEG 醛的平均分子量为 15 kDa 到 50kDa；

(b)反应产物的分离和纯化：在该反应过程中，蛋白质改性的程度用分子筛凝胶色谱进行监测，用含有 0.1M 氯化钠、pH 为 6.5 的 50mM 磷酸钠在 0.4 毫升/分钟的流速下进行洗脱：5 小时后，经分

子筛凝胶色谱法的分析表明所有的 hGH 已经基本上转化成为 N一末端聚乙二醇化的衍生物的形式；

随后用无菌水将该反应混合物总共稀释五倍，并且将其加到一个离子交换柱上，再用 pH 为 7.0 的 50mM 磷酸钠缓冲溶液饱和该柱；在 1 毫升/分钟的流速下向该柱中装填反应混合物，并且用三倍柱体积的同样的缓冲溶液洗脱未反应的 PEG 的醛；采用线性梯度洗脱 N一末端聚乙二醇化的 hGH，该线性梯度采用从 0%至 100%的 50mM 磷酸钠，其 pH 为 7.5、含 0.2M 氯化钠，进行 100 分钟，含有该 hGH 缀合物的部分被合并、浓缩并且无菌过滤。

通过烷基化的聚乙二醇化作用通常涉及在有一种还原剂存在下将 PEG 的一种醛的衍生物与 hGH 反应，通常生成的是多个 PEG 与 hGH 上的多个氨基酸偶联的缀合物。但人们可以通过控制该反应条件，从而基本上只使 hGH 的 N-末端 α -氨基上发生该聚乙二醇化反应，即一个单聚乙二醇化的物质，从而达到单一 PEG 分子连接到单一 hGH 分子上的效果。

通过还原性烷基化作用生产一种单聚乙二醇化产物的衍生化作用，利用了不同类型伯胺基的赖氨酸与 hGH 的 N-末端不同的反应性能，该伯胺基是在 hGH 的衍生化作用中可以利用的基团。上述反应是在 pH 值 3-9 下进行的，而该 PH 值可以让人们利用赖氨酸残基的 ε -氨基与 hGH 的 N-末端残基 α -氨基间 pKa 值的差异。通过这种选择性的衍生化作用，向一种多肽上连接含有一个活性基团的 PEG

是可以控制的。hGH 与该 PEG 的结合作用主要地发生在 hGH 的 N-末端上，而其它具反应活性的基团如赖氨酸侧链氨基，不发生明显的改性作用。在一个重要的方面，本发明提供了一种基本上均一的单 PEG/hGH 的结合物的分子制剂，而这表明 hGH 仅在一个单一的位置上被连接到一个聚合物分子上。更具体地讲，如果使用聚乙二醇的话，本发明也提供聚乙二醇化的 hGH，其可能缺少抗原连接基因并且含有被直接偶联到该 hGH 多肽上的聚乙二醇分子。

另外一个重要的考虑为聚合物与 hGH 的分子比。一般说来，该聚合物与 hGH 的分子比越低，可以连接到 hGH 上的聚合物分子的数目也越少。一般说来，对于此处所计划的聚乙二醇化反应而言，该聚合物优选的平均分子量为从 15kDa 至 50kDa。特别优选 40 kDa。hGH 与聚乙二醇的比值通常在 1：0.1 至 1：1 的范围内，优选 1：0.15 至 1：0.6。

pH 值也影响待用的聚合物与 hGH 之间的比值。一般说来，如果 pH 值较低，则需要聚合物：hGH 的比值量较大，即 N-末端 α-氨基的反应活性较差，则需要更多的聚合物来实现最佳反应条件。如果该 pH 值较高，则 PEG：hGH 的比值不需要如此之大，即有较多的反应活性基团可以利用，于是需要较少的聚合物分子。对于本发明的目的而言，该 pH 值通常落在 3-9 的范围内，优选 4.5-7。

通过采用如上所示的条件，按照本发明的还原性烷基化作用提供了一种连接方法，其中该连接为将 PEG 选择性地连接到任意一种

在氨基末端处具有一个α-氨基的 hGH 的多肽蛋白质上，并且提供了制备一种基本上为均一的单 PEG-hGH 缀合物。该 PEG-hGH 缀合物含有一个在 N－末端定位的 PEG 分子，而在 hGH 分子其它赖氨酸的侧链基团都不反应。上述制剂优选为生产出大于 60%的 PEG-hGH 缀合物，并且更优选生产出大于 70%的 PEG-hGH 缀合物，同时还伴有未反应的 PEG 及未反应的 hGH 分子。下面的实施例提供了一种至少有大约 70%的 PEG-hGH 缀合和与大约 70%的未反应的 hGH 的制剂。该 PEG-hGH 缀合物是具有生物学活性的。

对于本还原性烷基化作用而言，还原剂在液体状态下应当是稳定的，并且优选该还原剂能够仅还原在还原性烷基化作用的初期步骤中所形成的席夫碱 Schiff's base。优先的还原剂可以选自氢化钠、氰基硼氢钠，二甲胺甲硼烷三甲胺甲硼烷与吡啶甲硼烷所组成，特别优选的还原剂为氰基硼氢钠。

本发明也涉及聚乙二醇人生长激素缀合物在制备、治疗儿童或成人生长激素缺乏症的药物中的应用。

如上述所提及的，与本发明相应的 PEG-hGH 缀合物能够用于所有已知的天然 hGH 相同的用途，但注射频率由每天一次改为每 5-7 天一次。已有的临床应用已经表明 hGH 的临床效果，例如能促进人体充分利用各方面的能量和原料合成蛋白质，促进营养中的钙沉积在骨骼，增加身体细胞的大小和数量，使骨骼和内脏器官成比例增长，从而促进儿童身高的线性增长。

对于治疗用途而言，本发明的 PEG-hGH 缀合物可以配制于并且可以溶在任意一种无菌的生物相容的药用载体包括盐水，缓冲盐水及水中进行使用。在治疗疾病中发挥效用的 hGH 聚合物的用量依赖于上述疾病或状况的实质，并且该用量可以通过临床试验进行测定。当有可能时，人们首先在有用的动物体模型内测定本发明的 PEG-hGH 缀合物的疗效。注射的方法包括皮下，肌内给药。

使用剂量为 6mgPEG-hGH/kg 体重/次，每 5-7 天注射一次。

PEG-hGH 缀合物进行实际应用时，要保证它们在一段时间期限内的稳定性，并且还要便于患者使用，这就要加一些保护剂和稳定剂来保护 PEG-hGH 并制成特定的制剂。对 PEG-hGH 我们研究了液体剂型和冻干剂型两种剂型。

液体剂型的中各物质含量为每毫升含 30mg PEG-hGH，10mM 柠檬酸钠，4 mg 吐温 20，17.4 mg 氯化钠，苯酚 5 mg，pH6.0。

冻干剂型：先配成各物质含量为每毫升含 30mg PEG-hGH，甘氨酸 120mg，甘露醇 12mg，乳酸 12mg，碳酸氢钠 100 mg 的溶液，之后用冷冻干燥法制成冻干剂型，患者使用前加注射用水溶解。

在一个药效试验的实验例中，该方法包括将再溶解 PEG-hGH 的溶液引入生长激素缺乏动物体内的步骤。在该实验例中，所述的动物是大鼠，生长激素缺乏的大鼠是去垂体大鼠。

附图说明

附图名称是各组实验动物累计增重与时间关系曲线。

阴性对照组

hGH 组

40KGH 组

60KGH-30 组

60KGH-70 组

具体实施方式：

根据本发明的具体的 hGH 的制备及其生理学与生物学特性显示在下文中。所给出的这些实施例更为详尽地描述了本发明，但是并不打算用于限制本发明。在这些实施例中，hGH 均来源于基因重组技术。

实施例 1

连接位点在 N—末端 α —氨基处的

PEG40kDa—hGH 缀合物的制备

向溶于 50mM 磷酸钠，该溶液 pH 为 5.5 且含有 20mM $NaCNBH_3$，的 2.5 毫克/毫升 hGH 的一个冷却 4℃并搅拌的溶液中加入 0.3 倍摩尔的活化聚乙二醇的醛，该醛的平均分子量为 40000 道尔顿，即 40kDa。hGH 来自大肠杆菌表达技术生产。

在该反应过程中，蛋白质改性的程度用分子筛凝胶色谱进行监测，该色谱使用一个 Superose 6HR 10/30 柱，Pharmacia，用含有 0.1M 氯化钠、pH 为 6.5 的 50mM 磷酸钠在 0.4 毫升/分钟的流速下进行洗脱。5 小时后，经分子筛凝胶色谱法的分析表明所有的 hGH 已经基

本上转化成为N－末端聚乙二醇化的衍生物的形式。

随后用无菌水将该反应混合物总共稀释五倍，并且将其加到一个 HiLoad 16/10 S Sepharose HP 离子交换柱上，Pharmacia，再用 pH 为 7.0 的 50mM 磷酸钠缓冲溶液饱和该柱。在 1 毫升/分钟的流速下向该柱中装填反应混合物，并且用三倍柱体积的同样的缓冲溶液洗脱未反应的 PEG 的醛。采用线性梯度洗脱 N－末端聚乙二醇化的 hGH，该线性梯度采用从 0%至 100%的 50mM 磷酸钠，其 pH 为 7.5、含 0.2M 氯化钠，进行了 100 分钟，含有该 hGH 衍生物的部分被合并、浓缩并且无菌过滤。

实施例 2

连接位点在 N－末端 α 一氨基处的

PEG20kDa－hGH 结合物的制备

向溶于 50mM 磷酸钠，该溶液 pH 为 4.5 且含有 20mM $NaCNBH_3$ 的 2.5 毫克/毫升 hGH 的一个冷却 4℃并搅拌的溶液中加入 0.6 倍摩尔的活化聚乙二醇的醛，该醛的平均分子量为 20000 道尔顿，即 20kDa。hGH 来自哺乳动物表达技术生产。其余步骤重复实施例 1 的步骤。

实施例 3

连接位点在 N－末端 α 一氨基处的

PEG15kDa－hGH 结合物的制备

向溶于 50mM 磷酸钠，该溶液 pH 为 7.0 且含有 20mM $NaCNBH_3$

的 2.5 毫克/毫升 hGH 的一个 4℃并搅拌的溶液中加入 0.15 倍摩尔的活化聚乙二醇的醛，该醛的平均分子量为 15000 道尔顿，15kDa。hGH 来自哺乳动物表达技术生产。其余步骤重复实施例 1 的步骤。

实施例 4

连接位点在 N－末端 α －氨基处的

PEG50kDa－hGH 结合物的制备

向溶于 50mM 磷酸钠，该溶液 pH 为 6 且含有 20mM $NaCNBH_3$ 的 2.5 毫克/毫升 hGH 的一个 4℃并搅拌的溶液中加入 0.2 倍摩尔的活化聚乙二醇的醛，该醛的平均分子量为 50000 道尔顿，即 50kDa。hGH 来自大肠杆菌表达技术生产。其余步骤重复实施例 1 的步骤。

实施例 5

连接位点在 N－末端 α －氨基处的

PEG（30kDa）－hGH 结合物的制备

向溶于 50mM 磷酸钠，该溶液 pH 为 5.0 且含有 20mM $NaCNBH_3$ 的 2.5 毫克/毫升 hGH 的一个 4℃并搅拌的溶液中加入 0.4 倍摩尔的活化聚乙二醇的醛，该醛的平均分子量为 30000 道尔顿，即 30kDa。hGH 来自大肠杆菌表达技术生产。其余步骤重复实施例 1 的步骤。

实施例 6

液体剂型的制备：将实施例 1 方法制备的 PEG-hGH 1000mg 溶解于 330ml 液体剂型母液中，其中含 10mM 柠檬酸钠，4 mg 吐温 20，17.4 mg 氯化钠，苯酚 5 mg，pH6.0，无菌过滤后分装至 330 个 2ml

安瓶中，每瓶 1ml。

实施例 7

冻干剂型：将实施例 2 方法制备的 PEG-hGH 1000mg 溶解于 330ml 冻干剂型母液中，每 ml 母液中含有甘氨酸 120mg，甘露醇 12mg，乳酸 12mg，碳酸氢钠 100 mg 的溶液，无菌过滤之后，分装至 330 个 2ml 安瓶中，用冷冻干燥法制成冻干剂型，注射前加注射用水溶解。

实验例

利用摘除脑垂体的大鼠对 PEG－hGH 缀合物体内活性的评价

测定人生长激素产品活性的最经典方法之一是未成年去垂体大鼠的体重增加法（Body Weight Gain, BWG），已载入欧洲药典。

实验方法

实验用药:

hGH：原始样品每瓶含 0.5 克重组人生长激素，3.0 克赋形剂。用 Sephadex G25 脱盐柱缓冲体系变更为磷酸缓冲液（50mM, pH7.0）。首次给药前用载体稀释至 10mg/ml。

60KGH：60KGH 为 PEG_{40000}－hGH，原始浓度 0.088 毫克/毫升。首次给药前用载体稀释到 70 毫克/毫升。

40KGH：40KGH 为 PEG_{20000}－hGH，原始浓度 0.16 毫克/毫升。首次给药前用载体稀释至 70 微克/毫升。

纯度：

hGH：符合《中华人民共和国药典 2000 版》要求。

60KGH：PEG_{40000}－hGH 缀合物占 95%。

40KGH：PEG_{20000}－hGH 缀合物占 95%。

实验动物

Wister 品系大鼠（白求恩医科大学动物实验部），雄性，六周龄时将其脑垂体摘除。在脑垂体摘除后二周时，筛除体重增加速率超过标准差一倍的大鼠，共 53 只去垂体大鼠用于本研究，按照以下数量进行抽签分组。

阴性对照组大鼠 10 只；阳性对照组大鼠 10 只；60KGH（70 微克）给药组大鼠 11 只；60KGH（30 微克）给药组大鼠 11 只;40KGH（70 微克）给药组大鼠 11 只。

年龄：脑垂体摘除时为 6 周龄，开始给药时为 8 周龄。

接收时体重范围为 85.5 克至 111.6 克。

编号方法：每笼 2 只大鼠，用苦味酸饱合溶液在其中一只大鼠背部做标记，有标记者为 1 号码，无标记者为 0 号码，附加在笼号后面。示例：第 3 笼的大鼠编号为 30（无标记）和 31 号（有标记），第 10 笼大鼠编号为 100（无标记）和 101（有标记）。

给药

剂量和给药程序：

载体： 每次给予 1 毫升/大鼠，每天给予一次，连续给予 14 天。

hGH： 每次给予 10 微克/大鼠，每天给予一次，连续给予 14 天。

40KGH：每次给予 70 微克/大鼠，首日和第 8 日各给予一次。

60KGH：每次给予 70 微克/大鼠，首日和第 8 日各给予一次。

60KGH：每次给予 30 微克/大鼠，首日和第 8 日各给予一次。

给药途径：皮下注射。

体重测量

频率：给药前每 2-3 天测量一次。从首次给药起每天测量一次。

方法：使用电子台秤测量大鼠体重。

实验结果与结论

给药前大鼠体重日平均增长速率为 0.72g±0.36g

各组大鼠的平均体重为：

载体： 109.76g±6.60g

hGH: 110.86g±5.00g

40KGH: 107.85g±8.87g

60KGH(30μg): 112.78±7.24g

60KGH(70μg): 108.10g±5.73g

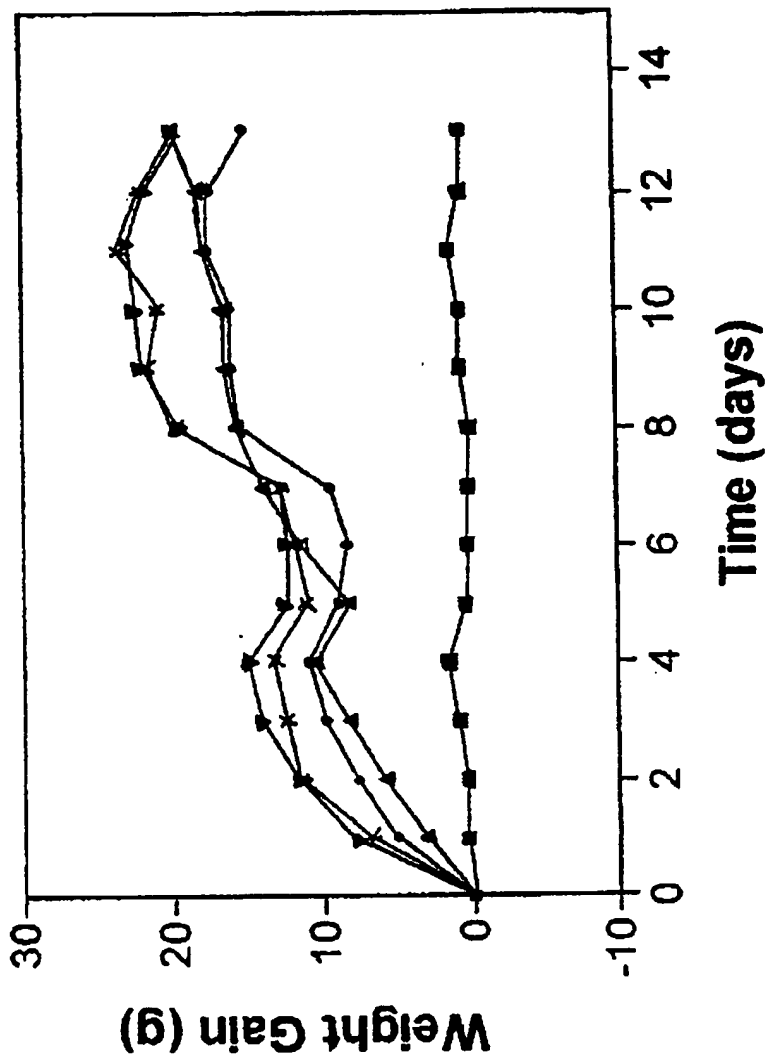
给药后各组大鼠体重每日平均累积增重（n 天体重－0 天体重）显示在图 1 中。

实验结果显示：

（1）阴性对照组（Vehicle）给药前后体重变化趋势一致，阴性对照成立；

（2）阳性对照组(hGH)给药前体重变化与阴性对照组和其他给药组一致；给药后日平均累积增重基本成直线上升，日体重增长速率均匀。因此阳性对照成立；

（3）70μg PEG－hGH 给药组（40KGH 和 60KGH）的日平均


Weight Gain (g)
30
20
10
0
-10
0
2
4
6
8
10
12
14
Time (days)

Appl. No. 02132612.6 Publ. No. 1468863A
Applicant: Wang Simian
Inventor: Wang Simian

PEG-hGH Conjugate, its Preparation & Medicinal Use

Field of the Invention

This invention relates to polyethylene glycolylated (PEGylated) protein, its preparation & medicinal use.

Background of the Invention

Ross clark et al disclose firstly the conjugation of human growth hormone (hGH) with PEG (MW 5kDa) and the result of its pharmacological activity studies ("The J. Biol. Chem. 271: 21969-21977, 1996). Said PEG-GH conjugate has markedly prolonged half-life, can be injected once per 5 days, and made convenient for the patient. However, said conjugated product is a mixture due to each GH molecule coupling to 1~7 PEG molecules, having indefinite coupling site & unquantifiable components, and hence unable to be used in clinical practice.

All kinds of native and recombinant proteins have medicinal uses. After purifying, isolating and formulating, they can be administered parenterally for different indication. However, the parenterally fed protein may be immunogenic, may be relatively water insoluble, and may have a shorter pharmacological half-life. Hence, it is difficult to make said proteins reach the therapeutic concn in patient blood. These difficult problems can be overcome by conjugating said protein to a polymer such as PEG. In US 4,179,337, Davis et al disclose a technical solution for coupling PEG to a protein (e.g. enzyme and insulin) to obtain a conjugate having an immunogenicity lower than the primary protein, and still maintaining the pharmocological activity in substantial proportion. In US 4,766,106 and 4,917,888, Katre et al also

disclose a technical solution for making the protein more soluble by compiling to polymer. Similarly, PEG and other polymers can be compiled to protein in order to decrease the immunogenicity and prolong the half-life.

A large number of medicinal PEGylated proteins are prepared, manufactured, and used in clinical practice along with the dev & deepness of biomedicine & gene Eng. There are PEGylated interferon、PEGylated granulocyte colony stimulating factor (G-CSF) etc.

Human growth hormone (hGH) is a peptide hormone secreted by human pituitary gland, human body is facilitated to fully utilize the energy & material from different aspects for synthesizing protein, the nutrient Ca^{2t} is facilitated to deposite on the bone, the size & quantity of body cell is increased, the human skeleton & internal organs is growth in proportion. In the year 1958, a foreign scientist extracted growth hormone from human pituitary gland to treat for dwarfism. The weight of human pituitary gland is about 0.5g, containing a minute quantity of hGH (5~10mg). The scientific researches were made extremely slow progress due to the limited source of the pituitary gland. In the year 1979, the expression of hGH's DNA sequence coding on E. Coli has been made possible by the DNA biotech, thereby the synthesized recombinant human growth hormone (rhGH) by gene Eng has been produced. The quality & yield of rhGH product is a very great improvement along with the uninterrupted growth of prod technique. In the year 1985, the synthesized rh GH by gene Eng came into use in clinical practice, brought about a good news for the patient. The patient must inject once daily owing to the short half-life of rh GH in vivo, it is very inconvenient to patient.

Disclosure of the Invention

For the feature of hGH's bare N-end, a PEG molecule is made to conjugate selectively to the hGH's N-end, and unreactable with other free arninogronps on h GH. The final product produced by this method is

monocomponent, easy to isolate & purified, and high yield due to each hGH molecule coupling to a single PEG molecule. The half-life of PEG-hGH is up to 5~7 days, and the medication frequency decreased (injecting once per 5~7 days), and made convenient for the patient.

This invention is a human growth hormone coupled to a polyethylene glycol (PEG. MW 15~50kDa), wherein each hGH molecule coupling to a single PEG molecule.

This invention is not limited to the PEG molecule of specific molecular weight, but the PEG has a single aldehyde group. In one embodiment, said PEG is PEG (4kDa). In a preferred embodiment, said hGH is made to conjugate to PEG (4kDa).

This invention's method for preparing PEG-hGH conjugate:

(a) Alkylation: adding hGH and activated PEG alaehyde into a solution (4°c, pH 3~9), the mol ratio of h GH to PEG being 1:0.1~1:1, the av. MW of PEG aldehyde being 15~50kDa;

(b) isolation and purification of the reaction product: monitoring the extent of protein modification in said reaction process by MS gel chromatography; eluting with 50 mM of a Na phosphate solution (pH 6.5, containing 0.1M of NaCl) at 0.4ml/min. After 5 hr, MS gel chromatographic analysis indicates: basically all the hGH has been converted into the form of N-end PEGylated derive.

Soon afterwards, diluting the reaction mixture with a septic water to five times its original volume, and applying to an ion exchange column; saturating said column with 50 mM of Na phosphate buffer solution (pH 7.0); filling the reaction mixture into said column at 1ml/min, and eluting the unreacted PEG aldehyde with three times the column volume of the same buffer solution; eluting the N-end PEG-hGH with linear gradient elution (using 50mM of a Na phosphate solution)(pH 7.5, containing 0.2M of NaCl, from 0% to 100%) for 100min; combining, concentrating and aseptic filtering the portions of

hGH conjugate.

The PEGylation by alkylation generally relates to a PEG aldehyde derive. Is made to react to hGH in the presence of a reactant, the product generally obtained is a multiple-PEG multiple-hHG conjugate. Nevertheless, byway of controlling said reaction condition, to make said PEGylation basically occur only at hGH's amino-N-end (I,e. mono PEGylated), there by a result of single PGE coupling to single hGH is achieved.

A derivatization for producing a mono PEGylate by reductive alkylation, has made use of the different reactive behavior of different type of lysine's prim. amino, with the hGH's N-end, said primary amino group is an utilizable group in the derivatization of hGH. The abovementioned reaction is performed at pH 3~9, and said pH value can be used for differencing the p Ka value between e-amino and α-amino (hGH N-end). Byway of this selective derivatization. It is controllable that conjugating a PEG (containing one active group) to a polypeptide. The PEG-hGH conjugation mainly occurs at hGH's N-end, and the marked modification not occurred at the other reactive group, e.g. Lysine-aminoof pendant chain. In a chief respect, this invention provides a molecular preparation of basically homogeneous mono PEG/hGH conjugate, and this indicates: hGH is conjugated to a polymer molecule only at a single position. More particularly, If PEG is used, this invention provides PEGylated hGH, being short of antigen joining gene and containing the PEG molecule coupling directly to said h GH polypeptide.

Another important consideration is the mol ratio of polymer to hGH. Generally, for the designed PEGylation herein, the preferred av. MW of said polymer is 15~50kDa, most preferably 40kDa. The mol ratio of hGH to PEG is generally 1:0.1~1:1, preferably is 1:0.15~1:0.6.

The pH value also has an influence on the ratio of polymer to be used to hGH. Generally, polymer is needed when pH value is lower: more polymer is required when the hGH ratio is high, to realize the maximum reaction

conditions. The ratio of PEG to hGH needs not so high when said pH value is higher, more reactive group can be used, and hence less polymer is needed. For the object of this invention, said pH value remains at 3~9, preferably at 4.5~7.

By using the conditions as shown above, according to this invention's reactive alkylation, a method for selectively conjugating PEG to any hGH polypeptide protein having an α-amino group at the N-end, and for preparing basically homogeneous mono PEG-hGH conjugate has been provided. Said PEG-hGH conjugate contains a PEG molecule located at N-end, and all other pendant chain groups of lysine are unreactable. Said preparation is preferably a>60% PEG-hGH conjugate, more preferably >70% PEG-Hgh conjugate, while accompanies unreacted PEG and hGH molecules. The following examples provide a preparaction containing at least ~ 70% of conjugate and ~ 70% of unreated hGH. Said PEG-Hgh conjugate possesses biological activity.

For this reactive alkylation, the reducing agent at liquid state should be stable, and preferably be capable of merely reducing the Schiff's base formed at the initial step of the reductive alkylation. The preferred reducing agent can be selected from the group consisting of Na hydride, Na cyanoborohydride, dimethylaminoborane, trimethylaminoborane, and pyridyl borane. The most preferred reducing agent is Na cyanoborahydride.

This invention also relates to the application of PEG-hGH to the pharmacy and therapeutics of growth hormoprivia (child or adult).

As shown above, the PEG-hGH correspondiong to this invention can be used for all the same known application as the native hGH, but the injection frequency is altered from once daily to once every 5~7 days. The clinic effect of hGH has been indicated by the prior clinic practice. For example, human body is facilitated to fully utilize the energy & material from different aspects for synthesizing protein, the nutrient ca^{2t} is facilitated to deposit on the bone, the size & quantity of body cell is ineuased, the human skeleton & internal

organs is grown in proportion, thereby the children's height is facilitated linear growth.

For the therapeutic applications, the PEG-hGH conjugate of the invention can be made preparations and dissolved in any aseptic biocompatible medical carrier, including saline, buffer saline and water, available for use. The effective dose of hGH polymer in curing diseases depends on the nature of said disease of state, and said dose can be determined through clinical test. If possible, firstly, the curative effect of this invention's PEG-hGH conjugate is determined in a model of an available animal body. The method of injection includes subcutaneous and intramuscular injections.

The therapeutical dose is 6 mg of PEG-hGH/kg wt once every 5~7 days.

In practical use, the stability of PEG-hGH in the space of several days must be ensured, and made convenient for the patient. Some protectant and stabilizer must be added to protect PEG-hGH and to be produced into specific preparation. For PEG-hGH, we have developed two forms of drug: liquid form and lyophilized form.

1 ml of the liquid form contains: 30mg of PEG-hGH, 10mM of Na citrate, 4mg of Tween 20, 17.4mg of NaCl, 5mg of phenol, pH 6.0.

the lyophilized form: firstly preparing a PEG-hGH -containing solution, then manufacturing said solution by lyophilization into lyophilized form, adding aseptic water before injection. 1 ml of said solution contains: 30mg of PEG-Hgh, 120mg of glycine, 12mg of mannitol, 12mg of lacticacid, 100mg of $NaHCO_3$.

In an example of pharmacokinetics, comprises the step of introducing the redissolved lyophilized form into the animal body of growth hormonoprivia, said animal being depituitary rats.

Brief Description of the Drawing

The name of the drawing is a plot of the cumulative weight gain for each group of experimental animal against the time.

■ Gp negative control

▲ Gp hGH ● Gp 60KGH-30

× Gp 40KGH ▼ Gp 60KGH-70

preferred Embodiments of the Invention

The following are the particular preparation and the physiological & biological features of hGH. It is intended to describe this invention in more detail, but never used in limiting this invention. In these examples, all hGH stems from gene recombination.

Example 1

Preparation of PEG (40kDa)-hGH (α-amino N-end) Conjugate

0.3nM of activated PEG aldehyde (av. MW 40kDa) was added into a 2.5mg/ml hGH solution [4°C, pH5.5, containing Nm of Hgh (E. coli expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring.

In said reaction process, the extent of protein modification was monitored by MS gel chromatography (Superose 6HR 10/30 column, Pharmacia), eluted with 50mM of a Na phosphate solution (pH 6.5, containing 0.1M of Nacl) at 0.4ml/min. After 5hr, MS gel chromatographic analysis indicated: basically all the hGH has been converted into the form of N-end PEGylated deriv.

Soon afterwards, the reaction mixture was diluted with a septic water to five times its original volume, and applied to HiLoad 16/10S Sepharose HP ion exchange column, Pharmacia, said column was saturated with 50mM of

Na phosphate buffer solution (pH 7.0). the reaction mixture was filled into said column at 1ml/min, and the unreacted PEG aldehyde was eluted with three times the column volume of the same buffer solution. The N-end PEGylated hGH was eluted with linear gradient elution using 50mM of a Na phosphated solution (pH 7.5, containing 0.2M of Nacl, from 0% to 100%) for 100 min; the portions of hGH conjugate were combined, concentrated, and aseptic filtered.

Example 2

Preparation of PEG (20kDa)-hGH (α-amino N-end) Conjugate

0.6nM of activated PEG aldehyde (av. MW 20kDa) was added into a 2.5mg/ml hGH solution [4°C, pH 4.5, containing nM of hGH (mammal expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring. For the rest, the steps of example 1 were repeated.

Example 3

Preparation of PEG (15kDa)-hGH (α-amino N-end) Conjugate

0.15nM of activated PEG aldehyde (av. MW 15kDa) was added into a 2.5mg/ml hGH solution [4°C, pH 7.0, containing nM of hGH (mammal expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring. For the rest, the steps of example 1 were repeated.

Example 4

Preparation of PEG (50kDa)-hGH (α-amino N-end) Conjugate

0.20nM of activated PEG aldehyde (av. MW 50kDa) was added into a 2.5mg/ml hGH solution [4°C, pH 6.0, containing nM of hGH (E. coli

expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring. For the rest, the steps of example 1 were repeated.

Example 5

Preparation of PEG (30kDa)-hGH (α-amino N-end) Conjugate

0.40nM of activated PEG aldehyde (av. MW 30kDa) was added into a 2.5mg/ml hGH solution [4°C, pH 5.0, containing nM of hGH (E. Coli expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring. For the rest, the steps of example 1 were repeated.

Example 6

Preparation of the Liquid Form

1000mg of the PEG-hGH produced from example 1 was dissolved in 330ml of the mother liquor of liquid form, wherein contains 10mM of Na citrate, 4mg of Tween 20, 17.4mg of Nacl, 5mg of phenol, pH 6.0. after aseptic filtering. The injecta was filled in 330 ampules (2ml).

Example 7

Preparation of the Lyophilized Form

1000mg of the PEG-hGH produced from example 2 was dissolved in 330ml of the mother liquor of lyophilized form. Each ml of the mother liquor contains 120mg of glycine, 12mg of mannitol, 12mg of lactic acid, 100mg of $NaHCO_3$. After aseptic filtering, said solution was filled in 330 ampules (2ml), and manufactured by lyophilization into lyophilized form, adding aseptic water before infection.

Expermental Example

Evaluation of In Vivo Activity on PEG-hGH Conjugate Using Depituitary Rats

One of the most classical method for assaying the activity of hGH is the Body Weight Gain BWG of the immature depituitory rats (written into European Pharmacopoeia).

Experimental method

Experimental Pharmacy:

hGH: primary sample, each ampule containing 0.5g of recombinant human growth hormone, 3.0g of excipient. The buffer system being altered to phosphate buffer (50mM, pH 7.0) using Sephadex G 25 desalting column. Diluting with a carrier to 10mg/ml before the first administration.

60KGH: being PEG (40kDa)-hGH, primary concn. 0.088mg/ml. diluting with a carrier to 70mg/ml before the first administration.

Purity:

hGH: according with "Chinese Pharmacopoeia 2000ed." Demands.

60KGH: 95% PEG (40kDa)

40KGH: 95% PEG (20kDa)

Experimental Animal

The rats of Wister strain (Animal Exp. Dept. Baiguien Univ.), male, 6 wk-age depituitary. In all 53 depituitary rats (screening higher rate of weight gain) are used in this studies. Drawing in groups according to the following numbers:

Gp negative control 10 rats	Gp 60KGH (70 μg) admn 11rats
Gp positiue control 10 rats	Gp 60KGH (30 μg) admn 11rats
	Gp 40KGH (70 μg) admn 11rats

Age: 6 wk-age when depituit, 8 wk-age when beg admn.

Range of body wt at received time 85.5~111.6g

Numbering Method: two rats in each cage wherein one rat is marked with a satd soln of picric acid. Typical examples: the rats in cage 3 numbered 30 (unmarked) and 31 (marked), the rats in cage 10 numbered 100 (unmarked) and 101 (marked).

Administration

The dose and procedure of admn:

Vehicle: 1ml/rat once, once daily for 14 days continuously

hGH: 1mg/rat once, once daily for 14 days continuously

40KGH: 70μg/rat once, once at first & eighth day each

60KGH: 70μg/rat once, once at first & eighth day each

60KGH: 30μg/rat once, once at first & eighth day each

Administration: subcutanious injection

Body Weight Measurement

Frequency: once per 2~3 days before admn, once daily from the first admn

Method: using electronic plat form scale

Experimental Result & Conclusion

The daily mean rate of rat's weight gain before admn is 0.72g±0.36g

The average weight of each group of rats:

Vehicle: 109.76g±6.60g

hGH: 110.86g±5.00g

40 KGH (70μg): 107.85g±8.87g

60 KGH (30μg): 112.78g±7.24g

60 KGH (70μg): 108.10g±5.73g

The daily mean cumulative weight gain of each group of rats after admn is shown in Fig. 1.

The experimental result indicates:

(1) Gp negative control (Vehicle): before & after admn, the weight variations are reaching unanimity, the negative control is tenable;

(2) Gp positive control (hGH): before admn, the weight variations are unanimous in the groups of negative control and other admn; after admn, the

daily mean cumulative weight gain sharp rises basically, and the daily mean rater of weight gain in creases uniformly, hence the positive control is tenable;

(3) The daily mean cumulative weight gain of Gps. 70μg PEG-hGH (40KGH and 60KGH)……

Claims

1. A polyethylene glycalylated human growth hormone (PEG-hGH) conjugate characterized by comprising an hGH conjugated to a polyethylene glycol having MW 15kDa, wherein each said hGH molecule coupling to a single PEG molecule.

2. The PEG-hGH conjugate of claim 1, characterized by said PEGylated hGH having a single reactive aldehyde group.

3. The PEG- hGH conjugate of claim 1, characterized by the hGH being coupled, through α-amino N-end, to PEG.

4. The PEG-hGH conjugate of claim 1, characterized by the molecular weight (MW) of PEG being 20kDa~40kDa.

5. The PEG-hGH conjugate of claim 1, characterized by said hGH being an expressed hGH molecule by gene engineering.

6. A method for preparing PEG- hGH conjugate, characterized by:

(a) Alkylation: adding hGH and activated PEG aldehyde into a solution (4°C, pH 3~9), the mol ratio of hGH to PEG being 1:0.1~1:1, the av MW of PEG aldehyde being 15~50kDa;

(b) isolation and purification of the reaction product: monitoring the extent of protein modification in said reaction process by MS gel chromatography; eluting with 50mM of a Na phosphate solution (pH 6.5, containing 0.1 M of NaCl) at 0.4ml/min. After 5hr, MS gel chromatographic analysis indicates: basically all the hGH has been converted into the form of N-end PEGylated derive.

Soon afterwards, diluting the reaction mixture with a septic water to five times its original volume, and applying to an ion exchange column; saturating said column with 50 mM of Na phosphate buffer solution (pH 7.0); filling the reaction mixture into said column at 1ml/min, and eluting the unreacted PEG aldehyde with three times the column volume of the same buffer solution;

eluting the N-end PEG- hGH with linear gradient elution (using 50 mM of a Na phosphate solution) (pH 7.5, containing 0.2M of NaCl, from 0% to 100%) for 10 min; combining, concentrating and aseptic filtrating the portions of hGH conjugate.

7. The method for preparing PEG-hGH conjugate of claim 6, characterized by when alkylate, the mol ratio of hGH to PEG being 1:0.5~1:0.6, the pH value being 4.5~7.

8. The application of PEG-hGH conjugate of claim 1 to the pharmacy and therapeutics of growth hormoprivia (child or adult).

Abstract

This invention relates to polyethylene glycolylated human growth hormone conjugate, its preparation & medicinal use. In said conjugate, each hGH molecule coupling to a single PEG molecule to prevent the rapid degradation of hGH in vivo, the polymer formed is more stable than hGH, and its in vivo half-life is markedly prolonged.

Appl. No. 02132612.6 Publ. No. 1468863A
Applicant: Wang Simian
Inventor: Wang Simian

PEG-hGH Conjugate, its Preparation & Medicinal Use

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This invention relates to polyethylene glycolylated (PEGylated) protein, its preparation & medicinal use.

Background of the Invention

Ross clark et al disclose firstly the conjugation of human growth hormone (hGH) with PEG (MW 5kDa) and the result of its pharmacological activity studies ("The J. Biol. Chem. 271: 21969-21977, 1996). Said PEG-GH conjugate has markedly prolonged half-life, can be injected once per 5 days, and made convenient for the patient. However, said conjugated product is a mixture due to each GH molecule coupling to 1~7 PEG molecules, having indefinite coupling site & unquantifiable components, and hence unable to be used in clinical practice.

All kinds of native and recombinant proteins have medicinal uses. After purifying, isolating and formulating, they can be administered parenterally for different indication. However, the parenterally fed protein may be immunogenic, may be relatively water insoluble, and may have a shorter pharmacological half-life. Hence, it is difficult to make said proteins reach the therapeutic concn in patient blood. These difficult problems can be overcome by conjugating said protein to a polymer such as PEG. In US 4,179,337, Davis et al disclose a technical solution for coupling PEG to a protein (e.g. enzyme and insulin) to obtain a conjugate having an immunogenicity lower than the primary protein, and still maintaining the pharmocological activity in substantial proportion. In US 4,766,106 and 4,917,888, Katre et al also

disclose a technical solution for making the protein more soluble by compiling to polymer. Similarly, PEG and other polymers can be compiled to protein in order to decrease the immunogenicity and prolong the half-life.

A large number of medicinal PEGylated proteins are prepared, manufactured, and used in clinical practice along with the dev & deepness of biomedicine & gene Eng. There are PEGylated interferon、PEGylated granulocyte colony stimulating factor (G-CSF) etc.

Human growth hormone (hGH) is a peptide hormone secreted by human pituitary gland, human body is facilitated to fully utilize the energy & material from different aspects for synthesizing protein, the nutrient Ca^{2t} is facilitated to deposite on the bone, the size & quantity of body cell is increased, the human skeleton & internal organs is growth in proportion. In the year 1958, a foreign scientist extracted growth hormone from human pituitary gland to treat for dwarfism. The weight of human pituitary gland is about 0.5g, containing a minute quantity of hGH (5~10mg). The scientific researches were made extremely slow progress due to the limited source of the pituitary gland. In the year 1979, the expression of hGH's DNA sequence coding on E. Coli has been made possible by the DNA biotech, thereby the synthesized recombinant human growth hormone (rhGH) by gene Eng has been produced. The quality & yield of rhGH product is a very great improvement along with the uninterrupted growth of prod technique. In the year 1985, the synthesized rh GH by gene Eng came into use in clinical practice, brought about a good news for the patient. The patient must inject once daily owing to the short half-life of rh GH in vivo, it is very inconvenient to patient.

Disclosure of the Invention

For the feature of hGH's bare N-end, a PEG molecule is made to conjugate selectively to the hGH's N-end, and unreactable with other free arninogronps on h GH. The final product produced by this method is

monocomponent, easy to isolate & purified, and high yield due to each hGH molecule coupling to a single PEG molecule. The half-life of PEG-hGH is up to 5~7 days, and the medication frequency decreased (injecting once per 5~7 days), and made convenient for the patient.

This invention is a human growth hormone coupled to a polyethylene glycol (PEG. MW 15~50kDa), wherein each hGH molecule coupling to a single PEG molecule.

This invention is not limited to the PEG molecule of specific molecular weight, but the PEG has a single aldehyde group. In one embodiment, said PEG is PEG (4kDa). In a preferred embodiment, said hGH is made to conjugate to PEG (4kDa).

This invention's method for preparing PEG-hGH conjugate:

(a) Alkylation: adding hGH and activated PEG alaehyde into a solution (4°c, pH 3~9), the mol ratio of h GH to PEG being 1:0.1~1:1, the av. MW of PEG aldehyde being 15~50kDa;

(b) isolation and purification of the reaction product: monitoring the extent of protein modification in said reaction process by MS gel chromatography; eluting with 50 mM of a Na phosphate solution (pH 6.5, containing 0.1M of NaCl) at 0.4ml/min. After 5 hr, MS gel chromatographic analysis indicates: basically all the hGH has been converted into the form of N-end PEGylated derive.

Soon afterwards, diluting the reaction mixture with a septic water to five times its original volume, and applying to an ion exchange column; saturating said column with 50 mM of Na phosphate buffer solution (pH 7.0); filling the reaction mixture into said column at 1ml/min, and eluting the unreacted PEG aldehyde with three times the column volume of the same buffer solution; eluting the N-end PEG-hGH with linear gradient elution (using 50mM of a Na phosphate solution)(pH 7.5, containing 0.2M of NaCl, from 0% to 100%) for 100min; combining, concentrating and aseptic filtering the portions of

hGH conjugate.

The PEGylation by alkylation generally relates to a PEG aldehyde derive. Is made to react to hGH in the presence of a reactant, the product generally obtained is a multiple-PEG multiple-hHG conjugate. Nevertheless, byway of controlling said reaction condition, to make said PEGylation basically occur only at hGH's amino-N-end (I,e. mono PEGylated), there by a result of single PGE coupling to single hGH is achieved.

A derivatization for producing a mono PEGylate by reductive alkylation, has made use of the different reactive behavior of different type of lysine's prim. amino, with the hGH's N-end, said primary amino group is an utilizable group in the derivatization of hGH. The abovementioned reaction is performed at pH 3~9, and said pH value can be used for differencing the p Ka value between e-amino and α-amino (hGH N-end). Byway of this selective derivatization. It is controllable that conjugating a PEG (containing one active group) to a polypeptide. The PEG-hGH conjugation mainly occurs at hGH's N-end, and the marked modification not occurred at the other reactive group, e.g. Lysine-aminoof pendant chain. In a chief respect, this invention provides a molecular preparation of basically homogeneous mono PEG/hGH conjugate, and this indicates: hGH is conjugated to a polymer molecule only at a single position. More particularly, If PEG is used, this invention provides PEGylated hGH, being short of antigen joining gene and containing the PEG molecule coupling directly to said h GH polypeptide.

Another important consideration is the mol ratio of polymer to hGH. Generally, for the designed PEGylation herein, the preferred av. MW of said polymer is 15~50kDa, most preferably 40kDa. The mol ratio of hGH to PEG is generally 1:0.1~1:1, preferably is 1:0.15~1:0.6.

The pH value also has an influence on the ratio of polymer to be used to hGH. Generally, polymer is needed when pH value is lower: more polymer is required when the hGH ratio is high, to realize the maximum reaction

conditions. The ratio of PEG to hGH needs not so high when said pH value is higher, more reactive group can be used, and hence less polymer is needed. For the object of this invention, said pH value remains at 3~9, preferably at 4.5~7.

By using the conditions as shown above, according to this invention's reactive alkylation, a method for selectively conjugating PEG to any hGH polypeptide protein having an α-amino group at the N-end, and for preparing basically homogeneous mono PEG-hGH conjugate has been provided. Said PEG-hGH conjugate contains a PEG molecule located at N-end, and all other pendant chain groups of lysine are unreactable. Said preparation is preferably a>60% PEG-hGH conjugate, more preferably >70% PEG-Hgh conjugate, while accompanies unreacted PEG and hGH molecules. The following examples provide a preparaction containing at least ~ 70% of conjugate and ~ 70% of unreated hGH. Said PEG-Hgh conjugate possesses biological activity.

For this reactive alkylation, the reducing agent at liquid state should be stable, and preferably be capable of merely reducing the Schiff's base formed at the initial step of the reductive alkylation. The preferred reducing agent can be selected from the group consisting of Na hydride, Na cyanoborohydride, dimethylaminoborane, trimethylaminoborane, and pyridyl borane. The most preferred reducing agent is Na cyanoborahydride.

This invention also relates to the application of PEG-hGH to the pharmacy and therapeutics of growth hormoprivia (child or adult).

As shown above, the PEG-hGH correspondiong to this invention can be used for all the same known application as the native hGH, but the injection frequency is altered from once daily to once every 5~7 days. The clinic effect of hGH has been indicated by the prior clinic practice. For example, human body is facilitated to fully utilize the energy & material from different aspects for synthesizing protein, the nutrient ca^{2t} is facilitated to deposit on the bone, the size & quantity of body cell is ineuased, the human skeleton & internal

organs is grown in proportion, thereby the children's height is facilitated linear growth.

For the therapeutic applications, the PEG-hGH conjugate of the invention can be made preparations and dissolved in any aseptic biocompatible medical carrier, including saline, buffer saline and water, available for use. The effective dose of hGH polymer in curing diseases depends on the nature of said disease of state, and said dose can be determined through clinical test. If possible, firstly, the curative effect of this invention's PEG-hGH conjugate is determined in a model of an available animal body. The method of injection includes subcutaneous and intramuscular injections.

The therapeutical dose is 6 mg of PEG-hGH/kg wt once every 5~7 days.

In practical use, the stability of PEG-hGH in the space of several days must be ensured, and made convenient for the patient. Some protectant and stabilizer must be added to protect PEG-hGH and to be produced into specific preparation. For PEG-hGH, we have developed two forms of drug: liquid form and lyophilized form.

1 ml of the liquid form contains: 30mg of PEG-hGH, 10mM of Na citrate, 4mg of Tween 20, 17.4mg of NaCl, 5mg of phenol, pH 6.0.

the lyophilized form: firstly preparing a PEG-hGH -containing solution, then manufacturing said solution by lyophilization into lyophilized form, adding aseptic water before injection. 1 ml of said solution contains: 30mg of PEG-Hgh, 120mg of glycine, 12mg of mannitol, 12mg of lacticacid, 100mg of $NaHCO_3$.

In an example of pharmacokinetics, comprises the step of introducing the redissolved lyophilized form into the animal body of growth hormonoprivia, said animal being depituitary rats.

Brief Description of the Drawing

The name of the drawing is a plot of the cumulative weight gain for each group of experimental animal against the time.

■ Gp negative control

▲ Gp hGH ● Gp 60KGH-30

× Gp 40KGH ▼ Gp 60KGH-70

preferred Embodiments of the Invention

The following are the particular preparation and the physiological & biological features of hGH. It is intended to describe this invention in more detail, but never used in limiting this invention. In these examples, all hGH stems from gene recombination.

Example 1

Preparation of PEG (40kDa)-hGH (α-amino N-end) Conjugate

0.3nM of activated PEG aldehyde (av. MW 40kDa) was added into a 2.5mg/ml hGH solution [4°C, pH5.5, containing Nm of Hgh (E. coli expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring.

In said reaction process, the extent of protein modification was monitored by MS gel chromatography (Superose 6HR 10/30 column, Pharmacia), eluted with 50mM of a Na phosphate solution (pH 6.5, containing 0.1M of Nacl) at 0.4ml/min. After 5hr, MS gel chromatographic analysis indicated: basically all the hGH has been converted into the form of N-end PEGylated deriv.

Soon afterwards, the reaction mixture was diluted with a septic water to five times its original volume, and applied to HiLoad 16/10S Sepharose HP ion exchange column, Pharmacia, said column was saturated with 50mM of

Na phosphate buffer solution (pH 7.0). the reaction mixture was filled into said column at 1ml/min, and the unreacted PEG aldehyde was eluted with three times the column volume of the same buffer solution. The N-end PEGylated hGH was eluted with linear gradient elution using 50mM of a Na phosphated solution (pH 7.5, containing 0.2M of Nacl, from 0% to 100%) for 100 min; the portions of hGH conjugate were combined, concentrated, and aseptic filtered.

Example 2

Preparation of PEG (20kDa)-hGH (α-amino N-end) Conjugate

0.6nM of activated PEG aldehyde (av. MW 20kDa) was added into a 2.5mg/ml hGH solution [4°C, pH 4.5, containing nM of hGH (mammal expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring. For the rest, the steps of example 1 were repeated.

Example 3

Preparation of PEG (15kDa)-hGH (α-amino N-end) Conjugate

0.15nM of activated PEG aldehyde (av. MW 15kDa) was added into a 2.5mg/ml hGH solution [4°C, pH 7.0, containing nM of hGH (mammal expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring. For the rest, the steps of example 1 were repeated.

Example 4

Preparation of PEG (50kDa)-hGH (α-amino N-end) Conjugate

0.20nM of activated PEG aldehyde (av. MW 50kDa) was added into a 2.5mg/ml hGH solution [4°C, pH 6.0, containing nM of hGH (E. coli

expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring. For the rest, the steps of example 1 were repeated.

Example 5

Preparation of PEG (30kDa)-hGH (α-amino N-end) Conjugate

0.40nM of activated PEG aldehyde (av. MW 30kDa) was added into a 2.5mg/ml hGH solution [4°C, pH 5.0, containing nM of hGH (E. Coli expression product), 50mM of Na phosphate, and 20mM of $NaCNBH_3$] while stirring. For the rest, the steps of example 1 were repeated.

Example 6

Preparation of the Liquid Form

1000mg of the PEG-hGH produced from example 1 was dissolved in 330ml of the mother liquor of liquid form, wherein contains 10mM of Na citrate, 4mg of Tween 20, 17.4mg of Nacl, 5mg of phenol, pH 6.0. after aseptic filtering. The injecta was filled in 330 ampules (2ml).

Example 7

Preparation of the Lyophilized Form

1000mg of the PEG-hGH produced from example 2 was dissolved in 330ml of the mother liquor of lyophilized form. Each ml of the mother liquor contains 120mg of glycine, 12mg of mannitol, 12mg of lactic acid, 100mg of $NaHCO_3$. After aseptic filtering, said solution was filled in 330 ampules (2ml), and manufactured by lyophilization into lyophilized form, adding aseptic water before infection.

Expermental Example

Evaluation of In Vivo Activity on PEG-hGH Conjugate Using Depituitary Rats

One of the most classical method for assaying the activity of hGH is the Body Weight Gain BWG of the immature depituitory rats (written into European Pharmacopoeia).

Experimental method

Experimental Pharmacy:

hGH: primary sample, each ampule containing 0.5g of recombinant human growth hormone, 3.0g of excipient. The buffer system being altered to phosphate buffer (50mM, pH 7.0) using Sephadex G 25 desalting column. Diluting with a carrier to 10mg/ml before the first administration.

60KGH: being PEG (40kDa)-hGH, primary concn. 0.088mg/ml. diluting with a carrier to 70mg/ml before the first administration.

Purity:

hGH: according with "Chinese Pharmacopoeia 2000ed." Demands.

60KGH: 95% PEG (40kDa)

40KGH: 95% PEG (20kDa)

Experimental Animal

The rats of Wister strain (Animal Exp. Dept. Baiguien Univ.), male, 6 wk-age depituitary. In all 53 depituitary rats (screening higher rate of weight gain) are used in this studies. Drawing in groups according to the following numbers:

Gp negative control 10 rats	Gp 60KGH (70 μg) admn 11rats
Gp positiue control 10 rats	Gp 60KGH (30 μg) admn 11rats
	Gp 40KGH (70 μg) admn 11rats

Age: 6 wk-age when depituit, 8 wk-age when beg admn.

Range of body wt at received time 85.5~111.6g

Numbering Method: two rats in each cage wherein one rat is marked with a satd soln of picric acid. Typical examples: the rats in cage 3 numbered 30 (unmarked) and 31 (marked), the rats in cage 10 numbered 100 (unmarked) and 101 (marked).

Administration

The dose and procedure of admn:

Vehicle: 1ml/rat once, once daily for 14 days continuously

hGH: 1mg/rat once, once daily for 14 days continuously

40KGH: 70μg/rat once, once at first & eighth day each

60KGH: 70μg/rat once, once at first & eighth day each

60KGH: 30μg/rat once, once at first & eighth day each

Administration: subcutanious injection

Body Weight Measurement

Frequency: once per 2~3 days before admn, once daily from the first admn

Method: using electronic plat form scale

Experimental Result & Conclusion

The daily mean rate of rat's weight gain before admn is 0.72g±0.36g

The average weight of each group of rats:

Vehicle: 109.76g±6.60g

hGH: 110.86g±5.00g

40 KGH (70μg): 107.85g±8.87g

60 KGH (30μg): 112.78g±7.24g

60 KGH (70μg): 108.10g±5.73g

The daily mean cumulative weight gain of each group of rats after admn is shown in Fig. 1.

The experimental result indicates:

(1) Gp negative control (Vehicle): before & after admn, the weight variations are reaching unanimity, the negative control is tenable;

(2) Gp positive control (hGH): before admn, the weight variations are unanimous in the groups of negative control and other admn; after admn, the

daily mean cumulative weight gain sharp rises basically, and the daily mean rater of weight gain in creases uniformly, hence the positive control is tenable;

(3) The daily mean cumulative weight gain of Gps. 70μg PEG-hGH (40KGH and 60KGH)……

Claims

1. A polyethylene glycalylated human growth hormone (PEG-hGH) conjugate characterized by comprising an hGH conjugated to a polyethylene glycol having MW 15kDa, wherein each said hGH molecule coupling to a single PEG molecule.

2. The PEG-hGH conjugate of claim 1, characterized by said PEGylated hGH having a single reactive aldehyde group.

3. The PEG- hGH conjugate of claim 1, characterized by the hGH being coupled, through α-amino N-end, to PEG.

4. The PEG-hGH conjugate of claim 1, characterized by the molecular weight (MW) of PEG being 20kDa~40kDa.

5. The PEG-hGH conjugate of claim 1, characterized by said hGH being an expressed hGH molecule by gene engineering.

6. A method for preparing PEG- hGH conjugate, characterized by:

(a) Alkylation: adding hGH and activated PEG aldehyde into a solution (4°C, pH 3~9), the mol ratio of hGH to PEG being 1:0.1~1:1, the av MW of PEG aldehyde being 15~50kDa;

(b) isolation and purification of the reaction product: monitoring the extent of protein modification in said reaction process by MS gel chromatography; eluting with 50mM of a Na phosphate solution (pH 6.5, containing 0.1 M of NaCl) at 0.4ml/min. After 5hr, MS gel chromatographic analysis indicates: basically all the hGH has been converted into the form of N-end PEGylated derive.

Soon afterwards, diluting the reaction mixture with a septic water to five times its original volume, and applying to an ion exchange column; saturating said column with 50 mM of Na phosphate buffer solution (pH 7.0); filling the reaction mixture into said column at 1ml/min, and eluting the unreacted PEG aldehyde with three times the column volume of the same buffer solution;

eluting the N-end PEG- hGH with linear gradient elution (using 50 mM of a Na phosphate solution) (pH 7.5, containing 0.2M of NaCl, from 0% to 100%) for 10 min; combining, concentrating and aseptic filtrating the portions of hGH conjugate.

7. The method for preparing PEG-hGH conjugate of claim 6, characterized by when alkylate, the mol ratio of hGH to PEG being 1:0.5~1:0.6, the pH value being 4.5~7.

8. The application of PEG-hGH conjugate of claim 1 to the pharmacy and therapeutics of growth hormoprivia (child or adult).

Abstract

This invention relates to polyethylene glycolylated human growth hormone conjugate, its preparation & medicinal use. In said conjugate, each hGH molecule coupling to a single PEG molecule to prevent the rapid degradation of hGH in vivo, the polymer formed is more stable than hGH, and its in vivo half-life is markedly prolonged.

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